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VALIDATED MICROBIOASSAY METHOD FOR ESTIMATION OF FREE AND ENCAPSULATED STREPTOMYCIN SULPHATE

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ABSTRACT

Streptomycin (STR), an antitubercular agent belongs to the class of aminoglycosides, which are deficient of a characteristic chromophore essential for spectrophotometric analysis. Complex or time consuming derivative spectroscopic methods or highly sophisticated techniques including HPLC, GC or LC-MS and immunoassays are thus being used for its detection and analysis including that in plasma.

Presently, we used the pharmacopoeial microbioassy method for its estimation in serum upon spiking the latter with free (F-STRS) or encapsulated in (solid lipid nanoparticles; STRS-SLNs) streptomycin sulphate (STRS). The standard curve (n=3) between log concentrations of STRS and diameter of zone of inhibition was plotted, and was found to be linear (r^2 = 0.958-0.978) over a range of recommended pharmacopoeial concentrations (0.64- 1.56 µg/ 0.1ml) in serum and also in phosphate buffer pH 8. Results were highly reproducible (RSD 2.9) and accurate (97%). The method was applied to estimate the total drug content and entrapment efficiency of STRS-SLNs. STRS is reported to loose its bioactivity at acidic pH probably due to ionisation. The same was not detected by colorimetric method. Using the presently developed method, it was observed that the loss of bioactivity of F-STRS in simulated gastric fluid (0.1N HCl; pH 1.2) was considerably reduced when STRS was incorporated into SLNs. Method could be, very suitably, extended to monitor the single dose (17mg/rat) pharmacokinetic studies in rats upon intramuscular administration of F-STRS.

Keywords: Solid lipid nanoparticles, Streptomycin, Microbioaasay, serum.

1. INTRODUCTION

Streptomycin (STR) is a water-soluble aminoglycoside derived from *Streptomyces griseus* and is marketed as the sulphate salt. Streptomycin sulphate (STRS) is a bactericidal antibiotic, acting by interfering with normal protein synthesis, active against a variety of organisms (both in vitro and in clinical infections) viz. *Brucella* (brucellosis), *Escherichia coli*, *Aerobacter aerogenes*, *Klebsiella pneumoniae*, and *Enterococcus faecalis* (in urinary tract infections), *Haemophilus ducreyi* (chancroid), *Haemophilus influenzae* (in respiratory, endocardial, and meningeal infections-concomitantly with another antibacterial agent), *Klebsiella pneumoniae* (pneumonia), *Mycobacterium tuberculosis*, *Pasteurella pestis*, *Streptococcus viridans*, and *Enterococcus faecalis* (in endocardial infections-concomitantly with penicillin). Since STR does not possess a strong UV-absorbing chromophore group, the state-of-the-art analytical method for its determination at low levels is liquid chromatography (LC) with post-column derivatization and fluorescence detection [1]. Latter is complex, costly and tedious and LC is an expensive equipment.

Microbiological assays have been used for the assay of antibiotics and are the methods of choice when an estimate of biological potency is required. Potency of an antibiotic is estimated by comparing the inhibition of growth of a sensitive microorganism, produced by known concentrations of the antibiotic being examined and a reference substance [2]. Microbiological assays are relatively inexpensive to perform, require little in the way of sophisticated equipment other than that used for handling the microorganisms and are suitable for testing large number of samples. The method can help to determine concentration of antibiotics even in less pure or crude samples without any elaborate extraction process being followed. In the present study we validated the microbioassay method, given in Indian Pharmacopoeia (IP), for determining concentration of STRS in aqueous samples and spiked serum samples. The method was used presently for (i) characterizing STRS-SLNs in terms of total drug content (TDC) and % entrapment efficiency (EE); (ii) comparing the efficacy/potency of STRS-SLNs in comparison to free STRS (F-STRS) in terms of degradation (loss of potency) induced at acidic pH of stomach; and (iii) intramuscular single dose pharmacokinetics studies in rats. F- STRS is administered parenterally by intramuscular route due to its poor and unreliable oral bioavailability. STRS was incorporated into SLNs with a proposal to enhance its biological performance (i.e. protection against gastric degradation) and oral bioavailability [3].

2. MATERIALS AND METHODS

STRS was obtained from Panacea Biotec, Lalru, India. Sodium hydroxide (Rankem, Mumbai), Antibiotic assay medium A (Hi-media Pvt. Ltd., India); *Bacillus subtilis* (MTCC 441); Potassium dihydrogen ortho phosphate (S.D. Fine Chem. Ltd., Mumbai, India); Soy Lecithin (Hi Media, India); Tween 80 (S.D. Fine Chemicals Ltd., India); Compritol® 888 ATO (Glyceryl Behenate, gift sample from Gattefosse, France) were also used in the study.

2.1. MICROBIOLOGICAL ASSAY OF STREPTOMYCIN

2.1.1. Preparation of the media

The prescribed dehydrated antibiotic assay medium A as per I.P and as detailed in table 1 was used for the studies.

2.1.2. Preparation of the sample solution

Five aqueous test dilutions (S1; 0.64, S2; 0.80, S3; 1, S4; 1.25, S5; 1.56 µg/0.1ml) of STRS in terms of the base streptomycin (STR) were prepared from the stock solution, with a ratio of 1:1.25 between successive dose levels. Median recommended dose of STR as per I.P is 1.0 µg/ml. Standard curve (n=3) prepared with one level assay was then obtained [4]

2.1.3. Test organisms

The test organism used for the test, as also recommended in I.P, was *Bacillus subtilis* with its identification number of MTCC 441.

2.1.4. Preparation of inoculum

Spore suspension of the *Bacillus subtilis* to be used for inocula was prepared in accordance to B.P. The organism was grown at 35°C - 37°C for 7 days on the surface of Assay medium A to which had been added 0.001g/l of manganese (II) sulphate (for inducing spore formation). The growth was washed off using sterile water. The suspension was heated at 70°C for 30 minutes (to kill the vegetative cells) and diluted to a concentration of 10^8 spores per ml. The cell count was confirmed using the pour-plate method. Briefly, the spore suspension was diluted 10^2 to 10^6 times and 1 ml each of these dilutions was added to sterile petri plates under aseptic conditions. Then 30 ml of nutrient agar media, at temperature not more than 45°C, was poured in the petri plates. The milk marketing board method was used for mixing the cell culture uniformly through out the plates [5]. Plates were kept at room temperature to

solidify, and were incubated, upside down, at 37°C for 17-18 hours, after which colonies were scored in plates showing 100 to 200 colonies. Cell count in original suspension was calculated by multiplying the obtained cfu with the corresponding dilution factor. Original spore suspension was then suitably diluted (based upon the determined spore count) to reach the required count of 10^8 cells/ml [6].

2.2. ONE-LEVEL ASSAY WITH STANDARD CURVE FOR STR

For preparing the standard curve, one-level assay with standard curve as described in IP was used with some modifications. Briefly 17 plates (×4 wells/ plate) were used to accommodate 68 wells which were suitably filled with various dilutions. Each plate accommodates 4 wells; 2 for median dose (S3) i.e. 1.0 µg/0.1ml and 2 for each of the remaining dilutions that is S1, S2, S4 and S5, respectively (Fig. 1). Four plates were used for each dilution (4x4 doses i.e. S1, S2, S4 and S5=16 plates), such that S3 was poured into 32 wells and each of the remaining concentration was poured into 8 wells (4 plates/concentration). All the four wells in the plate 17 were filled with S3, so as to result in a total of 36 readings for S3 as per recommendations of IP. The plates were incubated for 18 hours at 37°C and the zones of inhibition were measured using Vernier caliper.

Table 1 Dehydrated antibiotic assay medium A

Ingredients	**Quantity (g/1000 ml)**
Peptone	6.0
Pancreatic digest of casein	4.0
Yeast extract	3.0
Beef extract	1.5
Dextrose	1.0
Agar	15.0

Final pH of the media was 6.5-6.6 after sterilization

The readings of the S3 concentration tested on each set of 4 plate and plate 17 i.e. 36 reading were averaged. The average of the 36 readings of solution S3 was regarded as the correction point of the curve. The average value obtained for each concentration (S1, S2, S4 and S5) was then corrected in reference to S3 obtained for the four plates corresponding to each concentration. Taking the average of the 36 readings of solution S3 is the correction point for

the curve. Correct the average value obtained for each concentration (S1, S2, S4 and S5) to the figure it would be if the readings for solution S3 for that set of four plates were the same as the correction point. In correcting the value obtained with any concentration, say S1, if the average of 36 readings of S3 is, for example, 18.0 mm and the average of the S3 concentrations on one set of four plates is 17.8 mm, the correction is + 0.2 mm. If the average reading of S1 is 16.0 mm the corrected reading of S1is 16.2 mm. Corrected values thus obtained for the test concentration S1, S2, S3, S4 and S5 including the average of the 36 readings for solution S3 were then plotted on a two-cycle semi log graph, using the concentrations 0.64 µg/0.1ml to 1.56 µg/0.1ml (in terms of STR base) on ordinate and the diameters of the zones of inhibition as the abscissa, and an equation of best fit straight line was formed and was also compared to the line drawn through the points plotted for highest and lowest zone diameters obtained by means of the following expressions:

$$L = \frac{3a + 2b + c - e}{5}; \quad H = \frac{3e + 2d + c - a}{5}$$

Where, L = the calculated zone diameter for the lowest concentration of the standard curve response line; H = the calculated zone diameter for the highest concentration of the standard curve response line; c = average zone diameter of median dose readings of the reference point standard solution and a,b,d,e = corrected average values for the other standard solutions, lowest to highest concentrations, respectively [4].

However, for all practical purposes, wherever R^2 was ≥ 0.95 the former equation was used for determining the concentration of various samples.

Method was validated for linearity, accuracy and precision.

2.3. APPLICATION OF THE METHOD TO DETERMINE STRS IN STRS- SLNs

2.3.1. Using One Level Assay with Standard Curve method

For preparing the standard curve, the stock solution of STRS-SLNs (50 µg/ml as per colorimeteric assay method) [7] was prepared by dispersing STRS-SLNs in phosphate buffer pH 8. Further dilutions such as (SLN1; 0.64, SLN2; 0.80, SLN3; 1.0, SLN4; 1.25 and SLN5; 1.56 µg/0.1ml) were prepared by suitably diluting the stock solution with phosphate buffer pH 8.0. Twenty (20) petri plates with agar media were prepared to accommodate 80 wells. Two wells of each plate were filled with S3 (F-STRS = 1 µg/ml; as in previous assay) and other 2 wells were filled with a suitable SLN dilution (SLN1, SLN2, SLN3, SLN4 and SLN5;

4 plates/dilution). S3 concentration was used as the control and constituted of 1 µg free STRS/0.1 ml pH 8 phosphate buffer. Two wells of each plate were filled with S3 (40 wells) and remaining two wells with suitable SLN dilution, such that each SLN dilution was incorporated into 4 plates (8 wells). The plates were incubated for 18 hours at 37°C and the obtained zones of inhibition were measured using Vernier calliper. A zone diameter obtained for each plate were corrected in reference to S3 plated on the same plate and the average of readings of 40 wells containing S3. A straight response line was drawn for SLN1-5 as explained earlier.

2.3.2. Standard plot of F-STRS and STRS-SLNs in serum

The standard curve of F-STRS and STRS-SLNs was prepared by suitably diluting the respective stock solutions (50µg/ml) in serum to obtain various concentrations S1-S5 or SLN1-5, respectively. Twenty (20) petri plates were used to accommodate 80 wells to fill each dilution of STRS (40 wells of S3 in buffer and 8 each of S1-S5 concentrations after recovery from serum) or STRS-SLNS (40 wells with S3 in buffer and 8 each of SLN1-5 of recovered serum samples). Each plate accommodates 4 wells, 2 for median dose (S_3) and 2 for the respective dilutions of STRS or STRS- SLNS in serum, as obtained in the previous section. The plates were incubated for 18 hours at 37°C and the zones of inhibition were measured.

2.3.3. Recovery of STRS and STRS-SLNs in serum

Although significant zones of inhibition were obtained for serum samples spiked with F-STRS; but initial studies failed to elicit zones of inhibition for serum samples spiked with STRS-SLNs. When these samples were observed under optical microscope (Nikon Eclipse 90 i) clusters of these SLNs coated with proteins (albumin and apolipoproteins [8] could be observed (Fig 2). Latter may prevent the free diffusion of STRS from these aggregates, into agar. To overcome this problem the serum samples were treated with methanol to precipitate proteins and recover STRS from SLNs. Hence, it was thought appropriate to extract STRS from STRS-SLNs spiked serum samples and inoculated into the respectively labelled wells as explained in the previous section. Briefly, to 100 µl of the spiked serum samples in an ependorff tube, 200 µl of methanol was added and mixed well. Tubes were centrifuged at 10,000 rpm for 30 minutes at 4°C and supernatant (100 µl) was separated and inoculated into respectively labelled wells as explained in the previous section.

The plates were incubated for 18 hours, at 37°C, and concentration corresponding to the measured zones of inhibition calculated using the equation for the best fit line drawn for the respective standard curve of STRS or STRS SLNS in phosphate buffer. The values were substituted into the following equation to obtain recovery values.

$$\text{Recovery in serum} = \frac{observed\ concentration\ in\ serum}{actual\ concentration} \times 100$$

2.4. VALIDATION OF THE MICROBIOASSAY METHOD

2.4.1. Linearity

The standard plot using 5 different concentrations of STRS or STRS-SLNs was prepared in phosphate buffer pH 8 and serum, between log dose versus zone of inhibition and linearity was confirmed by determining r^2 value.

2.4.2. Accuracy

Accuracy of the assay procedure was determined by taking ratio of observed concentration and nominal concentration.

$$\text{Accuracy} = \frac{observed\ concentration}{nominal\ concentration} \times 100$$

2.4.3. Precision

The precision of the assay was determined by analyzing the lowest concentration of 0.64 µg/0.1ml prepared on 6 different occasions and the % CV was determined.

2.5. PREPARATION OF STRS-SLNS

STRS (0.727%), lipidic melt (Compritol® 888 ATO, 7.27 %, at 80°C), surfactant (polysorbate 80), co-surfactant (Soy-lecithin) and hot water, were mixed together to form a clear solution maintained at a 80˚C, under continuous stirring for 5 minutes. This solution was diluted with an equal quantity of cold water (2-4 ºC) to form SLN dispersion, under mechanical stirring at 5000 rpm. Stirring was continued for 1.5 hrs to result in a STRS loaded nanocolloidal dispersion (STRS-SLNs) [9, 10].

2.6. CHARACTERIZATION OF STRS-SLNs

2.6.1. Transmission electron microscopy (TEM)

Morphology of SLNs was examined using an electronic transmission microscope (Hitachi H-100; Japan).

2.6.2. Total drug content (TDC) and Entrapment Efficiency (EE) using Two-level factorial assay

Microbioassay method was followed to determine TDC and EE (n=3) using two level factorial assay. Parallel dilutions containing 2 levels of the standard STRS (S1; 2 µg/0.1ml and S2; 1 µg/0.1ml) prepared in phosphate buffer, at pH 8 and unknown concentration (test dilutions i.e of STRS-SLNs; U1 and U2) were prepared at same level and in a similar way. Each of the four wells of a petri plate (Fig: 3) were filled with different standard (S1 or S2) and test dilutions (U1 or U2). The plates were kept at room temperature for 30 min for diffusion and then incubated at 37º C for 17-18 hours. The diameters of the zone of inhibition were measured and percentage potency (TDC) of the sample was calculated as per the following equation:

Per cent potency = Antilog (2.0 + a log I)

Wherein a may have a positive or negative value and should be used algebrically.

$$\text{where } a = \frac{(U_1 + U_2) - (S_1 + S_2)}{(U_1 - U_2) + (S_1 - S_2)}$$

I = ratio of dilutions [4]

For percent EE determinations U1 and U2 were prepared by suitably the pellet obtained by ultracentrifuging the STRS-SLNs at a speed of 8.02 lac g, in a cold ultra centrifuge (Beckman Coulter optima L100K ultracentrifuge), in sterile water.

2.7. DETERMINATION OF BIOACTIVITY OF STRS AND STRS-SLNs IN 0.1 N HCl

This was also done using two level factorial assay design as explained above. The method involved preparation of standard STRS concentrations (S1: 2 µg/0.1ml; S2: 1 µg/0.1ml) in phosphate buffer, pH 8 and the same concentrations ($U1_{HCL}$: 2 µg/0.1ml; $U2_{HCL}$; 1 µg/0.1ml) in 0.1 N HCl taken as unknown (U) concentrations. The standard concentrations (S1 and S2) were refrigerated (4-8°C) while the unknown samples were maintained at 37°C for 4 hrs. Aliquots (0.1ml) were withdrawn at different time interval 0 min, 15 min, 30 min, 1 hour, 2 hours and 4 hours from both the standard and unknown samples and were suitably inoculated into wells of a plate. The plates were kept at room temperature for diffusion of dilutions for 30 minutes and were then incubated at 37°C for 17-18 hours. Test was performed in triplicate for each time point. The potency was then calculated by the equation discussed earlier. The

method was similarly extended to STRS-SLNs ($S_{1SLN\ and}$ S_{2SLN}) using $U1_{SLN,\ HCl}$ and $U2_{SLN,\ HCl}$ as the unknown concentration exactly as explained above. Both the S_{SLN} and $U_{SLN,\ HCL}$ concentrations were incubated at 37℃ and samples were withdrawn at suitable time intervals.

2.8. PHARMACOKINETIC APPLICABILITY OF THE VALIDATED MICROBIOLOGICAL METHOD FOR DETERMINATION OF STRS IN SERUM AFTER INTRAMUSCULAR ADMINISTRATION

2.8.1 Animals:

Male Wistar rats weighing 200-210 gm, bred in Central Animal House facility of Panjab University were used for the study. Animals were housed (3/cage) in polyacrylic cages [38×23×10cm] and maintained under standard laboratory conditions with natural dark and light (approximately 12:12 h) cycle and had free access to standard pellet diet and water. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

2.8.2. Treatment design:

In order to evaluate the pharmacokinetic applicability of the validated microbiological assay, a single dose of 17 mg/rat (rats with weight between 200-210 gm were selected for study) of F-STRS was administered intramuscularly to the rats. Blood was withdrawn at time intervals of 15 min, 30 min, 1 hour, 2 hour, 4 hour and 8hour from retro-orbital plexus of rats (n=4). Animals were sacrificed at the end of the study. The obtained blood samples were stored at room temperature to separate serum. 100 µl of serum at each time point was incorporated into 2 wells of a petri plate; adding S3 concentration of standard STRS in other two wells of the same petri plate. Zones of inhibition obtained for each time point were corrected suitably using the corresponding zones of inhibition values obtained for S3 and the averaged S3 values of the standard plot. Concentration of STR in serum sample of each time point was then read from the constructed standard plot of F-STRS in serum using the corresponding diameter of zone of inhibition at each time point.

2.8.3. **Dose selection**

Recommended human dose of STR is 1g = 1000 mg/70 Kg (adult human wt.); for a rat of 200 mg and at 6 times higher dose (fast metabolism rate in rats, versus humans as per FDA recommendations)[11], the dose/rat = 17 mg/rat.

3. RESULTS

Microbiological method for the estimation of STRS in phosphate buffer pH 8 and in rat serum was developed and validated according to the principles of Good Laboratory Practices.

Table 2 Recovery of STRS (determined as STR) from serum and STRS-SLNs

Spiked amount of drug µg/0.1ml	% Recovery	
	Serum	In Phosphate buffer
0.64	98.3	101
0.80	107.8	102
1.0	102.9	100
1.25	97.7	95
1.56	104.1	105
Mean	102.1	100.6
SD	±4.03	±3.64

3.1. Standard plot

The standard curve (n=3) between log concentration of STRS and STRS-SLNs (as free base STR) versus diameter of zone of inhibition was plotted, and was found to be linear (r^2 = 0.958-0.978) over the range of prepared concentrations (0.64-1.56 µg/ 0.1ml) in serum and also in phosphate buffer pH 8 (Fig 4-7).

3.2. VALIDATION OF MICROBIOLOGICAL METHOD OF ANALYSIS FOR STREPTOMYCIN (STRS)

Recovery of STRS (as STR) from STR-SLNs was found to be satisfactory (Table 2). The absolute recovery of STRS, after spiking the rat serum with various concentrations (0.64, 0.8, 1, 1.25, 1.56 µg/0.1ml in terms of STR) was in the range of 97.7-107.8%. Accuracy of the microbioassay method for determining STRS (as STR) in phosphate buffer pH 8 and in rat serum both from free STRS and STRS-SLNS was found to be sufficiently high for pH 8 buffer and rat serum respectively (Table 3). Precision (% CV) for the assay was found to be 2.9 % in phosphate buffer pH 8 and 1.96 % in rat serum (Table 4).

Table 3 Accuracy of determining the concentration of STRS (as STR) in phosphate buffer pH 8 and in serum (n=3)

S. No	Nominal Conc. (µg/0.1 ml)	Diameter (mm)		Average Experimental		% Accuracy	
		Phosphate Buffer	**Serum**	**Phosphate Buffer**	**Serum**	**Phosphate Buffer**	**Serum**
1	0.64	11.3 ± 0.71	11.6 ± 0.78	0.59	0.58	92.1	90
2	0.80	12.0 ± 0.85	12.6 ± 0.7	0.76	0.82	95.0	102
3	1.0	13.1 ± 0.96	13.6 ±0.64	1.02	1.05	102.0	105
4	1.25	14.4 ± 0.84	14.6 ± 0.43	1.31	1.28	104.8	102
5	1.56	14.9 ± 0.55	15.5 ± 0.75	1.43	1.49	91.6	95.5
Mean						97.1	98.9

Table 4 Precision of determining a 0.64 µg/ 0.1ml concentration of STR as STRS in phosphate buffer and serum (n=3)

S. No	Diameter (mm)	
	Phosphate buffer	**Serum**
1	11.8	11.6
2	11.5	11.5
3	12.0	11.6
4	11.6	12.1
5	12.6	12.0
6	12.0	11.6
7	11.8	11.5
8	11.6	11.6
Mean	11.9	11.7
SD	±0.4	±0.2
% CV	2.9	1.96

3.3. TEM:

When observed under TEM, STRS-SLNs were found to be spherical in shape. The size of the nanoparticles under TEM was observed to be in the range of 100-150 nm (Fig. 8).

3.4. TDC and % EE of STRS-SLNs using the above validated method

The developed microbioassay method was suitably applied to determine TDC and % EE of the developed SLNs. The values were found to be 90.56± 2.1% and 54.48±1.7 % respectively.

3.5. BIOACTIVITY OF F-STRS AND STRS-SLNs IN 0.1 N HCL

The acid degradation studies were performed to evaluate the protection provided by SLNs against the degradation effects of gastric acid, pH 1.2. The results were compared with the values obtained after incubating same concentrations of the standard drug in phosphate buffer pH 8. It was found that 50% (Table 6) of F-STRS degraded after incubation at 4 hours at 37°C in 0.1 N HCl. In contrast more than 95 % of STRS -SLNs remained intact after 4 hours of incubation under similar conditions. The results are indicative of the protective asylum provided to STRS by the lipid core of SLN, which prevents the degradation and maintains the bioactivity of STRS in acidic medium.

3.6. STR CONCENTRATION IN SERUM AFTER ADMINISTRATION OF STRS BY INTRAMUSCULAR (i.m) ROUTE:-

Pharmacokinetic study was performed after administration of single intramuscular dose of 17mg of F-STRS/rat (Table 7). Free drug concentration was detected in serum for up to 2 hours post administration of drug and C_{max} was 64.39 µg/ml; at 0.25 h (t_{max}). Free drug could not be detected at later time points, indicating its fast clearance of from the body.

4. DISCUSSION

Several microbioassay methods have been reported for estimation of streptomycin in buffer and serum in earlier part of the 20th century, claiming the method to be simpler and not involving the use of costly reagents or equipments to perform them [12]. The rugged and robust nature of these tests has endured more than 65 years, starting with reports as early as 1945 when minimum specification for streptomycin was established by FDA [13, 14] and methods for its determination in body fluids were reported using *B.subtilis.* Some workers also reported on use of *S.aureus* and modified assay methods using glass tubes [15], capillaries [16], paper discs to get results with in 5-6 hrs [12] and much smaller antibiotic volume (0.02 ml) with error < ± 5% [17].

The microbiological assay of an antibiotic is based upon a comparison of the inhibition of growth of micro-organisms by measured concentration of the antibiotics under examination with that produced by known concentrations of a standard preparation of the antibiotic having

a known activity. The cylinder/well plate method depends upon diffusion of the antibiotic from a vertical cylinder/well through a solidified agar layer in a petri dish or plate to an extent such that growth of the added microorganism is prevented entirely in a zone around the cylinder/well containing a solution of the antibiotic. Presently we used the well established Pharmacopeial method (I.P) for determining STR in buffer and in serum and the results were confirmed for their accurateness and precision. The procedure was found to show CV < 3.0% and 2.0% respectively. The validated microbiassay method was extended to estimate the total drug content and entrapment efficiency of STRS-SLNs. The method was found applicable for determination of STR (in serum and buffer) when applied as STRS-SLNs with significant accuracy (100.6 and 102.1) respectively. STRS-SLNs have been developed with an intent to explore non-invasive oral route of administration for streptomycin in place of the conventional parenteral (intramuscular) route (results not shown). The microbioassay method was suitably applied to determine TDC and % EE of the developed SLNs. The values were found to be 90.56±2.1% and 54.48±1.7% (n=6) respectively and were in agreement with the previously reported analytical colorimetric method [7]. The acid degradation studies were performed to evaluate the protection provided by SLNs against the degradation effects of gastric acid (pH 1.2). The results were compared with the values obtained after incubating same concentrations of the standard drug in phosphate buffer pH 8. It was found that SLNs (only 5% degradation) provide significant protection to free STRS which showed 50% degradation when incubated with 0.1M HCl within (4 hours at 37°C). However this loss in bioactivity was not manifested in colorimetric determinations probably because the derivatised group which is being determined by analytical procedures does not alter at acidic pH. It is reported that the loss of bioactivity of STR at acidic pH is due to ionization. The microbioassay methods always have an advantage for monitoring the concentration in terms of bioactivity which is highly relevant for antibiotics or other antimicrobial substances.

In order to evaluate the pharmacokinetic applicability of the validated microbiological assay, a single intramuscular dose of 17 mg of STRS/rat, was administered. C_{max} obtained for F-STRS at 0.25 h was 64.39 µg/ml. Results are in agreement with the pharmacokinetic data reported for STR [18].

Table 5 Accuracy of determining STRS-SLNs in phosphate buffer pH 8 and serum

S.No	Nominal Conc. (µg/0.1 ml)	Diameter (mm)		Average Experimental Conc.		% Accuracy	
		Phosphate Buffer	**Serum**	**Phosphate Buffer**	**Serum**	**Phosphate Buffer**	**Serum**
1	0.64	11.6±0.14	10.8 ± 0.1	0.58	0.64	90	100
2	0.80	12.6±0.21	11.4 ± 0.2	0.80	0.76	100	95
3	1.0	13.8±0.14	12.5 ± 0.01	1.07	0.98	107	98
4	1.25	14.6±0.07	14.2 ± 0.1	1.25	1.33	100	106
5	1.56	15.7±0.07	15.0 ± 0.1	1.50	1.49	96	95
Mean						99	102

Table 6 % remaining bioactivity of STRS-SLNs and STRS (n=3) upon incubation in 0.1 N HCl for different times

Time	% Remaining	
	STRS	**STRS-SLNs**
0 min	100 ± 0.2	100 ± 0.16
15 min	98.2 ± 0.12	100 ± 0.12
30 min	98.1 ± 0.15	100 ± 0.16
60 min	85.0 ± 0.09	100 ± 0.18
2 hrs	76.5 ± 0.11	95.9 ± 0.14
4 hrs	50.0 ± 0.10	95.5 ± 0.16

Table 7 Concentration of free STRS in serum after intramuscular administration

Time	Concentration µg/ml
0 min	0 ± 0
15 min	64.39 ± 0.96
30 min	56.8 ± 0.61
60 min	49.5 ± 0.88
2 hrs	42.2 ± 0.61
4 hrs	-
8 hrs	-

- Not detected

5. CONCLUSIONS

A sensitive and specific microbiological assay method was developed and validated presently. The standard curve between log concentrations of streptomycin and diameter of zone of inhibition was plotted, and was found to be linear over a range of recommended pharmacopoeial concentrations (0.64- 1.56 µg/ 0.1ml) in serum and also in phosphate buffer pH 8. The present method was extended to evaluate the loss of bioactivity of F-STRS in comparison to STRS-SLNs upon incorporation into simulated gastric fluid (0.1N HCl; pH 1.2) for varying periods of time. The method was applied to estimate the total drug content and entrapment efficiency of STRS-SLNs. The method was preliminarily applied to determine the concentration of STRS in serum after intramuscular administration of F-STRS, respectively upon administration by intramuscular route. It may be concluded, that the method can be extended to pharmacokinetic studies, upon oral administration of STRS loaded SLNs. Latter has scope for a more favourable treatment of tuberculosis

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